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**FERTILITY IMPAIRING VACCINE CONTAINING AVIAN ZONA
PELLUCIDA PROTEIN AND METHOD OF USE**

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Background of the Invention

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Population control of both domesticated and wild, free-roaming animals has been a major problem around the world. Overpopulation in dogs and cats results in millions of euthanasias of unwanted pets yearly in the United States alone, and overpopulation of wild animals causes destruction of habitat and threatens other species within the ecosystem. In humane shelters population control of unwanted pets is currently achieved through euthanasia of the animals. In general, after capture, dogs are held for a period of one week. If they are not adopted, they are humanely destroyed.

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Finding a socially acceptable as well as a safe and effective means of controlling excess populations of animals has been extremely difficult. Traditional methods of population control in dogs have been largely unsuccessful. Surgical spaying is a laborious procedure, requiring the initial induction of the animal, gas anesthesia during surgery, a surgical pack with suture materials and post-operative medications. Common surgical complications include problems associated with the procedure itself, allergic reactions to anesthetics or post-operative medications, and adverse local or systemic effects during the recovery period. Examples include ovarian remnant syndrome, where dogs continue to cycle despite being spayed, uterine infections, abdominal hemorrhage, and premature opening of the suture line. A substantial recovery period is typically needed even after an uncomplicated procedure.

Surgical spaying is also expensive, and pet owners are often unwilling to assume the costs.

The use of a reversible contraceptive is an attractive alternative to surgical spaying. However, conventional hormonal therapies that have been used to curb pet overpopulation are inconvenient because they usually require daily administration of the drug. Furthermore, most protracted hormonal therapies have undesirable side effects such as uterine infections, mammary cancer, and diabetes.

A vaccine comprising porcine zona pellucida protein (pZP) and an adjuvant comprising synthetic trehalose dicorynomycolate has been successfully used to cause immunocontraception in horses (P. Willis et al., *J. Equine Vet. Sci.* 14:364-370 (1994)) and elephants (R. Fayer-Hosken, *Wildlife Soc. Bull.*, 25(1):18-21 (1997)). Injection of total heat solubilized pZP into horses results in an immunocontraceptive effect that lasts for about 12 months and has been effective in reducing fertility of free-roaming horses (Kirkpatrick et al., *J. Reprod. Fert.* 94:437-444 (1992)). The pZP vaccine has been used for more than 8 years in horses with no adverse reactions; nor does the vaccine affect the feti of pregnant mares.

A pZP vaccine has been administered to dogs as well, albeit with somewhat less success. Mahi-Brown et al. have shown that infertility in the female dog can be achieved by vaccinating with a crude pZP preparation, but vaccination was accompanied by abnormal estrus cycles and other deleterious side effects, such as ovarian cyst formation (*J. Exp. Zool.*, 222:89-95 (1982); *Am. J. Reprod. Immunol. Microbiol.*, 18: 94-103 (1988)).

Porcine zona pellucida protein is obtained from pig ovaries, thus the amount of vaccine that can be prepared is limited by the supply of pig ovaries. Dunbar et al. (e.g., EP 599822, U.S. Pat. No. 5,637,300) have experimented with reproductive control in non-rodent mammals using a recombinant zona pellucida protein. However, due to limitations imposed by recombinant DNA technology and available expression systems, the recombinant protein lacks the glycosylation pattern of the native glycoprotein and its immunogenicity has not been satisfactorily demonstrated. What is clearly needed to combat overpopulation of domesticated and wild, free-roaming

animals is a low cost contraceptive or sterilant that possesses a high degree of efficiency, lacks of harmful side effects, is amenable to remote delivery, requires a minimal number of administrations, and is easy to produce.

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Summary of the Invention

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The present invention provides a fertility impairing vaccine and a method for impairing fertility in an animal. One embodiment of the fertility impairing vaccine, referred to herein as a protein vaccine, comprises a polypeptide comprising at least one avian zona pellucida protein or an immunogenic fragment thereof. These proteins are related to the mammalian zona pellucida proteins. Optionally, the protein vaccine of the invention further includes a polypeptide comprising a porcine zona pellucida protein or an immunogenic fragment thereof.

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Another embodiment of the fertility impairing vaccine, referred to herein as a polynucleotide vaccine, comprises a polynucleotide comprising at least one nucleotide sequence that encodes a polypeptide comprising an avian zona pellucida protein or an immunogenic fragment thereof. Optionally, the polynucleotide vaccine of the invention further includes a polynucleotide comprising a nucleotide sequence that encodes a polypeptide comprising a porcine zona pellucida protein or an immunogenic fragment thereof.

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The fertility impairing vaccine of the invention optionally further includes an immunological adjuvant or immunostimulant, preferably synthetic trehalose dicorynomycolate (STDCM). Also optionally, the fertility impairing vaccine contains an oil, preferably squalene oil.

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The method for impairing fertility in the animal comprises administering to the animal the fertility impairing vaccine of the invention in a manner and an amount effective to cause fertility impairment in the animal.

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When administered as an immunocontraceptive, the fertility impairing vaccine causes temporary, reversible infertility in the animal. When administered as an immunosterilant, the fertility impairing vaccine causes permanent, irreversible infertility in the animal. In dogs and cats, for example, immunosterilization is

far preferable to surgical sterilization and hormone regimens as a population control tool.

The fertility impairing vaccine of the invention can generally be used for immunological control of fertility and reproduction in any desired animal population, whether domestic or wild. As an example, the fertility impairing vaccine can be used to control ferrel dog and cat populations by development of a species-specific oral delivery vehicle. As another example, the fertility impairing vaccine can be used to control wild horse, deer or elephant populations when formulated as a single dose dart or bullet that can be administered without capture or confinement of the animal.

Detailed Description of the Preferred Embodiments

The fertility impairing vaccine of the invention contains a polypeptide comprising at least one avian zona pellucida protein (aZP) or an immunogenic fragment thereof, or a polynucleotide having a nucleotide sequence encoding a polypeptide comprising an avian zona pellucida protein or an immunogenic fragment thereof. The avian zona pellucida protein is preferably, but need not be, substantially pure. The avian zona pellucida protein or immunogenic fragment thereof can be a naturally occurring protein, a chemically or enzymatically synthesized protein, or a recombinant protein. The avian zona pellucida protein or immunogenic fragment thereof is preferably, but need not be, glycosylated. In a glycosylated avian zona pellucida protein (i.e., an avian zona pellucida glycoprotein), the glycosylation pattern is preferably equivalent to the glycosylation pattern found on a native (i.e., naturally occurring) glycoprotein. Naturally occurring avian zona pellucida protein is preferably obtained from a chicken. Avian zona pellucida protein can be conveniently obtained from bird ovaries or from an egg cell of the bird and/or the surrounding extracellular matrix and tissue, particularly the perivitelline membrane, for example from an oocyte or unfertilized egg of a bird at any stage of development.

In a preferred embodiment of the fertility impairing vaccine, the avian zona pellucida protein used in the vaccine or encoded by the

polynucleotide used in the vaccine is a total avian zona pellucida protein. A total avian zona pellucida protein preparation includes a plurality of immunoreactive proteins, some of which have been identified in chickens as gp42 and gp97 (Y. Takeuchi et al., *Eur. J. Biochem.* 260:736-742 (1999)), and gp34 and gp95 (M. Waclawek et al., *Biol. Reprod.* 59:1230-1239 (1998)).
5 Example I, below, identifies immunoreactive chicken proteins having molecular weights of 70 kD, 40 kD and 35 kD. The fertility impairing vaccine can, alternatively, contain fewer than all the avian zona pellucida proteins; for example it can contain the 40 kD and the 35 kD proteins but not the 70 kD protein.
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Purity of the avian zona pellucida protein or glycoprotein can be evaluated analytically using a combination or series of two-dimensional polyacrylamide gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with both silver staining and Coomassie Blue staining, and Western blot analysis, as described in the following Examples. Glycoproteins typically migrate electrophoretically in gels as broad smears rather than narrow bands, as a result of the variable levels of negative charge present in the constituent oligosaccharide chains. A total avian zona pellucida protein preparation isolated from bird eggs typically migrates as three distinct smears in the gel electrophoretic experiments, and shows the immunological reactivity in Western blot analysis using a polyclonal antibody raised in rabbits to highly purified total porcine zona pellucida protein. In a substantially pure avian zona pellucida protein preparation, no contaminating proteins having electromigration patterns different from those exhibited by the avian zona pellucida proteins are detectable in the two-dimensional gel, the SDS-PAGE gel, or Western blot analyses.
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An immunogenic fragment of an avian zona pellucida protein or glycoprotein is a peptide fragment, preferably a glycosylated peptide fragment, that elicits an immune response in a subject to which it is administered. An immune response includes either or both of a cellular immune response or production of antibodies, and can include activation of the subject's B cells, T cells, helper T cells or other cells of the subject's immune system. For example,
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an immune response is evidenced by a detectable anti-aZP antibody level in the subject using ELISA substantially as described in Example III.

5 Immunogenicity of an avian zona pellucida protein fragment can be determined, for example, by administering the adjuvanted candidate fragment to the subject, then observing of the associated immune response by analyzing anti-aZP fragment antibody titers in serum. An immunogenic peptide fragment preferably contains more than seven amino acids, more preferably at least about 10 amino acids, most preferably at least about 20 amino acids.

10 In a preferred embodiment of the fertility impairing vaccine, the polypeptide used in the vaccine or encoded by the polynucleotide used in the vaccine further includes at least one epitope, such as a T cell, helper T cell or B cell epitope. The epitopes can be derived from the species to which the vaccine is to be administered, from the species that was the source of the zona pellucida protein or immunogenic fragment thereof, or from any other species, including a virus, bacterium, or parasite. T cell, helper T cell or B cell epitopes or epitope mimics have been identified in zona pellucida proteins (Garza et al., J. Reprod. Immunol., February 1998, pp. 87-101). The use of immune cell epitopes derived from an immunogenic organism, such as a pathogenic parasite, is preferred. For example, the polynucleotide can encode a chimeric peptide comprising a T cell or helper T cell epitope from a parasite and a B cell epitope from a zona pellucida protein, such as a porcine or avian zona pellucida protein (Bagavant et al., Biol. Reprod., March 1997, pp. 764-770). A vaccine for use as an immunosterilant preferably contains a polypeptide that contains at least one T cell epitope (or a polynucleotide functionally encoding such a polypeptide),
20 whereas a vaccine for use as an immunocontraceptive preferably includes a polypeptide that encodes at least one B cell epitope (or a polynucleotide functionally encoding such a polypeptide).

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30 A polynucleotide encoding the polypeptide comprising the avian zona pellucida protein or immunogenic fragment thereof can include DNA, RNA, a modified nucleic acid, or any combination thereof. The polynucleotide can be supplied as part of a vector or as a "naked" polynucleotide. General methods for construction, production and administration of polynucleotide vaccines are known in the art, e.g. F. Vogel et al., Clin. Microbiol. Rev. 8:406-

410 (1995). Polynucleotides can be generated by means standard in the art, such as by recombinant techniques, or by enzymatic or chemical synthesis.

A polynucleotide used in a fertility impairing vaccine of the invention is preferably one that functionally encodes an avian zona pellucida protein. A protein is "functionally encoded" if it is capable of being expressed from the genetic construct that contains it. For example, the polynucleotide can include one or more expression control sequences, such as *cis*-acting transcription/translation regulatory sequences, including one or more of the following: a promoter, response element, an initiator sequence, an enhancer, a ribosome binding site, an RNA splice site, an intron element, a polyadenylation site, and a transcriptional terminator sequence, which are operably linked to the coding sequence and are, either alone or in combination, capable of directing expression in the target animal. An expression control sequence is "operably linked" to a coding sequence if it is positioned on the construct such that it does, or can be used to, control or regulate transcription or translation of that coding sequence. Preferred expression control sequences include strong and/or inducible *cis*-acting transcription/translation regulatory sequences such as those derived from metallothionein genes, actin genes, myosin genes, immunoglobulin genes, cytomegalovirus (CMV), SV40, Rous sarcoma virus, adenovirus, bovine papilloma virus, and the like.

The coding and expression control sequences for the avian zona pellucida protein are preferably constructed in a vector, such as a plasmid of bacterial origin, a cosmid, episome, or a viral vector, for administration to a target animal. A vector useful in the present invention can be circular or linear, single-stranded or double stranded. There are numerous plasmids known to those of ordinary skill in the art useful for the production of polynucleotide vaccine plasmids. A specific embodiment employs constructs using the plasmid pcDNA3.1 as the vector (InVitrogen Corporation, Carlsbad, CA). In addition, the vector construct can contain immunostimulatory sequences (ISS) that stimulate the animal's immune system. Other possible additions to the polynucleotide vaccine constructs include nucleotide sequences coding cytokines, such as granulocyte macrophage colony stimulating factor (GM-CSF) or interleukin-12 (IL-12). The cytokines can be used in various combinations to

fine-tune the response of the animal's immune system, including both antibody and cytotoxic T lymphocyte responses, to bring out the specific level of response needed to affect the animal's reproductive system.

5 Alternatively, the vector can be a viral vector, including an adenovirus vector, and adenovirus associated vector, or a retroviral vector. Preferably the viral vector is a nonreplicating retroviral vector such as the Moloney murine leukemia virus (N2) backbone as described by Irwin et al. (*J. Virology* 68:5036-5044 (1994)).

10 Optionally, a fertility impairing protein vaccine of the invention further includes a polypeptide comprising a porcine zona pellucida protein or an immunogenic fragment thereof. The porcine zona pellucida protein or immunogenic fragment thereof can be a naturally occurring protein, a chemically or enzymatically synthesized protein, or a recombinant protein. The porcine zona pellucida protein or immunogenic fragment thereof is preferably, but need not be, glycosylated. In a glycosylated porcine zona pellucida protein (i.e., a porcine zona pellucida glycoprotein), the glycosylation pattern is preferably equivalent to the glycosylation pattern found on a native (i.e., naturally occurring) glycoprotein. Immunogenicity of the porcine zona pellucida fragment is as generally described for avian zona pellucida protein.

15 20 Without intending to be bound by any particular theory or mechanism, the porcine zona pellucida protein or immunogenic fragment thereof can be included in the fertility impairing vaccine as an adjuvant to enhance the recipient's immune response, or for its independent and/or synergistic effect in impairing fertility in the recipient. For example, a fertility impairing protein vaccine substantially comprising chicken zona pellucida protein, which is readily available and relatively inexpensive to isolate, can be augmented by the addition of a small amount of porcine zona pellucida protein, which is more difficult and expensive to obtain. Notwithstanding the above, any desired ratio of avian zona pellucida protein to porcine zona pellucida protein can be used in this embodiment of the vaccine of the invention. The relative amounts of avian zona pellucida protein and porcine zona pellucida protein used in this embodiment of the vaccine depend on the nature of the animal being vaccinated and the immunogenic response generated in the animal by the vaccine. Preferably the

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ratio of avian zona pellucida to porcine zona pellucida (aZP: pZP) used in this embodiment of the vaccine is between 100:1 and 1:100.

Similarly, a polynucleotide vaccine of the invention optionally includes, in addition to a polynucleotide encoding a avian zona pellucida protein or immunogenic fragment thereof, a polynucleotide encoding a porcine zona pellucida protein or an immunogenic fragment thereof. The porcine zona pellucida polynucleotide functionally encodes porcine zona pellucida protein and can take the form of various genetic constructs as generally described for the polynucleotide encoding avian zona pellucida protein. Polynucleotides encoding avian and porcine zona pellucida proteins can constitute part of the same vector or can be delivered to the recipient on different vectors.

The fertility impairing vaccine of the invention, whether it contains a polypeptide (e.g., conjugated or non-conjugated avian zona pellucida protein) or a polynucleotide encoding a polypeptide, optionally further includes an immunological adjuvant to enhance the immunological response of the subject to the immunogenic polypeptide or polynucleotide. Examples of adjuvants include Freund's Complete Adjuvant, Freund's Incomplete Adjuvant, Freund's mycotoxin free adjuvant, aluminum hydroxide, EQUIMUNE (a deproteinized highly purified cell wall extract derived from non-pathogenic *Mycobacteria* spp., Acemannan (a long-chain polydispersed β (1,4) linked mannan polymer interspersed with O-acetylated groups, permulum, and an adjuvant comprising an immunostimulant such as synthetic trehalose dicorynomycolate (STDCM). The vaccine can also optionally include an oil, such as squalene oil, drakeol, or vegetable oil, which can also have an adjuvant effect (see P. Willis et al., J. Equine Vet. Sci., 14, 364-370 (1994)). It should be noted, however, that companion birds exhibit a sensitivity to oil-based adjuvants, which cause necrotic granulomas at the site of administration. An adjuvant comprising synthetic trehalose dicorynomycolate, squalene oil, and a surfactant such as lecithin is preferred for use in mammals. Lecithin typically includes phosphatidyl choline. An adjuvant comprising aluminum hydroxide is preferred for use in birds.

When an oil adjuvant is used, homogenization of the adjuvant, such as Freund's adjuvant, with the aqueous zona pellucida protein or polynucleotide

5 solution can be accomplished using any convenient means known in the art, such that the oil disperses within the aqueous solution to form an oil in water emulsion. Oil droplet sizes of about 200 nm or less are particularly preferred as they produce a more uniform and stable suspension. A particularly preferred fertility impairing vaccine comprises a predetermined amount of zona pellucida protein or polynucleotide and a pharmaceutically acceptable immunogenic level of adjuvant in an emulsion containing about 10% oil phase and about 90% aqueous phase.

10 In another embodiment of the invention, the fertility impairing vaccine, whether it contains a conjugated or a non-conjugated avian zona pellucida protein or a polynucleotide encoding said polypeptide, contains no adjuvant; essentially the fertility impairing vaccine of this embodiment is an aqueous avian zona pellucida protein or polynucleotide solution that delivers the intended amount of avian zona pellucida protein or polynucleotide to the 15 recipient.

20 In a preferred embodiment of the protein vaccine for use in mammals, the vaccine comprises oil, preferably a biodegradable oil such as squalene oil, in an amount of about 2.5% to about 15%, preferably about 8% to about 12%. In preparing the composition it is advantageous to combine a concentrated oily adjuvant composition with an aqueous solution of the immunogen, aZP protein. Typically, the composition is prepared using an adjuvant concentrate which contains lecithin (about 5% to about 15 % wt/vol, preferably about 12% wt /vol) and STDCM (preferably about 25 mg/mL to about 50 mg/mL) in squalene oil. The term % wt/vol means grams per 100 mL 25 of liquid. The aqueous solution containing the isolated aZP protein is typically a phosphate-buffered saline (PBS) solution, and additionally preferably contains Tween 80 (about 0.2% vol/vol to about 0.8% vol/vol, preferably about 0.4% vol/vol). See J.A. Rudbach et al., "Ribi Adjuvants: Chemistry, Biology and Utility in Vaccines for Human and Veterinary Medicine," in The Theory and 30 Practical Application of Adjuvants, D.E.S. Stewart-Tull, Ed., John Wiley & Sons, New York, NY (1995)). Homogenization of the oily adjuvant concentrate with the aqueous aZP solution can be accomplished using any convenient means known in the art, such that the oil disperses within the aqueous solution to form

an oil in water emulsion. Oil droplet sizes of about 200 nm or less are particularly preferred as they produce a more uniform and stable suspension. A particularly preferred vaccine comprises predetermined amounts of aZP and STDCM in an emulsion containing about 10% squalene oil and about 90% aqueous phase.

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The fertility impairing vaccine is administered in a manner effective to cause impaired fertility in an animal. An "animal," as that term is used herein, is an oocyte-producing organism that is capable of voluntary mobility. Preferred animals include mammals and birds. More preferably, the animal is a mammal; most preferably it is a deer, horse, elephant, rat, mouse, rabbit, ferret, cat or dog. Birds include but are not limited to free-ranging birds and domesticated birds, including food birds such as chickens, turkeys, and waterfowl, and companion birds in the order Psittaciformes, Passeriformes, Columbiformes, Falconiformes, such as budgerigars, canaries, finches, cockatiels, lovebirds, pigeons, doves and hawks.

Impairment of fertility in an animal in accordance with the invention can take the form of either immunocontraception and immunosterilization. Immunosterilization means permanent, irreversible infertility, in contrast to immunocontraception wherein infertility is temporary or transient, and reversible. Immunocontraception and immunosterilization are both dependent on the antibody titer level in the serum of the subject, but immunosterilization is typically the result of ovarian pathology caused by the immune response of the animal, as evidenced by, for example, total destruction of the zona pellucida proteins and/or influx of leukocytes into the follicles. Reducing the number of boosters leads to reduced immune response which results in immunocontraception (i.e., infertility that is temporary and reversible) instead of immunosterilization.

Optionally, the zona pellucida protein can be conjugated to an immunogenic carrier protein. A conjugated form of an avian zona pellucida protein is suitable for use in embodiments of the fertility impairing vaccine containing an avian zona pellucida protein that is homologous with respect to the intended recipient; that is, where the avian zona pellucida protein is isolated from the same species to which it is to be administered. For example, where a

fertility impairing vaccine comprising chicken zona pellucida protein is intended for administration to chickens, it may be desirable to conjugate the chicken zona pellucida protein to an immunogenic carrier to augment the immune response of the recipient. However, it is to be understood that the invention includes administration of avian zona pellucida protein from any avian source to any bird, whether homologous or heterologous, and whether the avian zona pellucida protein is conjugated or unconjugated. For example, the invention includes administration of a fertility impairing vaccine comprising an unconjugated chicken zona pellucida to psittacine birds.

25 A polynucleotide vaccine is preferably administered in one or more doses containing the plasmid, viral vector or naked polynucleotide in an amount of about 5 μ g to about 100 μ g. One of skill in the art can readily determine a suitable dosage for a particular animal, depending on the nature, size and overall health of the animal, as well as the condition to be treated.

For vaccines administered to mammals, an immunostimulant such as STDCM is typically present in a per dose amount of about 50 μ g to about 5 mg, preferably in an amount of about 200 μ g to about 3.5 mg, more preferably in an amount of about 400 μ g to about 3.0 mg. For vaccines administered to birds, an immunostimulant such as STDCM is optionally present in a per dose amount of about 10 μ g to about 5 mg, preferably in an amount of about 50 μ g to about

3.5 mg, more preferably in an amount of about 1 mg to about 3 mg. When administered to a bird, the fertility impairing vaccine preferably contains ALOH as an adjuvant, or contains no adjuvant at all.

An initial injection of the vaccine is followed by one or more booster injections at two to four week intervals, although the boosters can be administered from about 9 days to about twelve months following the previous vaccination. Preferably, two booster shots are administered following the initial administration. The body's immunological response to the vaccine at this dosing regimen is expected to render the ovaries permanently inactive as a result of, for example, follicle disruption or destruction, as evidenced by immunocytochemical analysis and histological evaluation of the ovarian tissue of vaccinated subjects. Sterility is permanent and irreversible.

Immunosterilization of animals in accordance with the present method is not expected to cause abnormal estrus cycles or other significant undesirable side effects in the vaccinated subjects.

When the fertility impairing vaccine is administered to a dog or a cat as described above, but with only one booster instead of two or more boosters, the vaccine is expected to result in immunocontraception (i.e., temporary or transient, reversible infertility) rather than immunosterilization.

Fertility impairment in dogs in accordance with the present method preferably is not expected to cause abnormal estrus cycles or other significant undesirable side effects in the vaccinated subjects.

Although the fertility impairing vaccine is typically administered by way of intramuscular injection, other forms of administration are also contemplated, including subcutaneous or intradermal administration, oral administration, as by food or water, topical administration, including transdermal administration, aerosol administration, cloacal or vaginal administration, intracoelomic administration, intranasal administration, and transconjunctival administration, including the use of eye drops. In addition, liposome-mediated, microsphere-mediated, and microencapsulation systems are all included as delivery vehicles for the fertility impairing vaccine of the present invention. The fertility vaccine can be formulated as a single dose vaccine and packaged for delivery through a dart or a bullet, such that it can be administered

to an animal from a distance. A single shot time-release delivery vehicle is also contemplated, wherein the avian zona pellucida protein or immunogenic fragment thereof (or the polynucleotide encoding the avian zona pellucida or immunogenic fragment thereof) is supplied in a single dose in two or more 5 different forms, such as a free protein and a microencapsulated protein, to allow administration of the immunogenic agent to the animal over a longer period of time.

Advantages of the invention are illustrated by the following 10 examples. However, the particular materials and amounts thereof recited in these examples, as well as other conditions and details, are to be interpreted to apply broadly in the art and should not be construed to unduly restrict or limit the invention in any way.

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EXAMPLES

Example I. Isolation of Avian Zona Pellucida Proteins

Perivitelline membranes were obtained from laid chicken eggs. 20 Yolks were separated from whites by making a small hole in the egg and draining the albumin. The yolks were removed from the shell and the chalazae was removed and discarded. The perivitelline membranes (pvm) were either manually peeled away or punctured to drain the yolk, and the membranes were washed in sterile phosphate buffered saline solution. A tissue homogenizer 25 (Powergamer 700D) was used to homogenize the membranes. M. Waclawek et al. (Biol. Reprod., **59**, 1230-1239 (1998)) reported a similar procedure for isolating perivitelline membranes from laid eggs that can also be used. Alternatively, perivitelline membranes can be isolated directly from ovarian follicles by preparing granulosa cell sheets substantially as described by A. Gilbert et al, J. 30 Reprod. Fertil., **50**, 179-181 (1977)).

Purity was demonstrated and confirmed using one-dimensional and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis combined with Western blot analysis, silver staining, and, at times, Coomassie

blue staining, using standard protocols. Proteins having molecular weights of 70 kD, 40 kD and 35 kD reacted with rabbit anti-pZP serum. Yolk and albumin controls did not react with the rabbit anti-pZP serum. The 70 kD protein was easily washed away from the perivitelline membrane. The 35 kD protein was approximately twice as abundant as the 40 kD protein and both had strong reactivity to the anti-pZP serum. One or both of these proteins are likely to be homologous to mammalian ZP3 (also known as ZPC), according to published reports (Y. Takeuchi et al., Eur. J. Biochem., 260, 736-742 (1999); M. Waclawek et al., Biol. Reprod., 59, 1230-1239 (1998)).

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Example II. Preparation of Avian Zona Pellucida (aZP) Vaccine

The vaccine is prepared by homogenizing a concentrated oily adjuvant concentrate with an aqueous antigen solution containing isolated aZP protein. The oily adjuvant concentrate contains a surfactant, lecithin, and an immunostimulant, synthetic trehalose dicorynomycolate (STDCM), in squalene oil. A typical adjuvant concentrate contains about 12% wt/vol (grams/100 mL) lecithin and about 25-50 mg/mL STDCM in squalene oil. The aqueous antigen solution contains the aZP protein preparation in saline or phosphate buffered saline (PBS) and Tween 80. When prepared for use in combination with an adjuvant concentrate to yield the vaccine composition, the aqueous composition typically contains 0.4% (vol/vol) Tween 80 and an amount of aZP calculated to yield a dose of about 100 μ g to about 400 μ g per vaccination. Vaccine doses for dogs are about 1 mL in volume.

Homogenizing is accomplished by combining adjuvant concentrate (to a final concentration of no greater than 10% vol/vol) with aqueous aZP solution and emulsifying using a Powergam 700D homogenizer at 15,000 rpm for 6 minutes. The resulting emulsion is then homogenized with phosphate buffered saline (PBS) (containing 0.4% vol/vol Tween 80) at 20,000 for 8-12 minutes. The homogenization process results in a vaccine composition that is an oil-in-water emulsion or possibly a water-in-oil-in-water emulsion. While the inventors do not intend that the invention be bound by any particular scientific theory, it is believed that the STDCM, an amphiphilic glycolipid, partitions to

the oil/water interfaces in the emulsion, and that the antigen is attracted to and associates with the STDCM at these interfaces.

Example III. Immunosterilization of Dogs using aZP Vaccine

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Vaccinations. Female dogs are vaccinated with 200 μ g of aZP per dose in a vaccine adjuvanted with synthetic trehalose dicorynomycolate (STDCM, commercially available from RIBI Immunochem Co., Hamilton, MT) in squalene oil. The amount of STDCM per dose was about 100 μ g to about 2.5 mg. A 10X adjuvant concentrate as described in Example II is available from RIBI Immunochem Co., Hamilton, MT, and vaccines are prepared as described in Example II. Boosters (containing the same amount of aZP, 200 μ g) are administered at 14-day intervals. Vaccinations are delivered to dogs intra-muscularly in the longissimus muscle (loin area). Booster injections are administered on the contra-lateral side.

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Antibody titers. Blood is drawn from each dog weekly, and serum antibody titers are determined using an enzyme linked immunosorbant assay (ELISA). Adjacent wells of a microwell plate are coated with 2 μ g aZP, and incubated for 6 hours. The wells are then blocked with 5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in TBST (Tris-buffered saline + 5% Tween-20) and incubated overnight. Wells are then loaded with the primary antibody (canine serum) in TBST at a 1:500 and 1:1,000 dilution and incubated for 4 hours. The wells are then washed and loaded with 50 μ l of the secondary antibody (rabbit anti-dog IgG) and incubated for 2 hours. Color change is observed after the addition of *p*-nitrophenyl phosphate for 30 minutes and the reaction terminated by the addition of 3 M NaOH. The optical density is read at a 405-492 nm range on a Spectramax spectrophotometer. The dog's pre-immune serum serves as the negative control.

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Example IV. Administration of Mixed Chicken/Porcine ZP Vaccine to Psittacines

Vaccinations. Chickens are vaccinated with 200 µg of mixed zona pellucida protein (1:1, aZP:pZP) per dose (1 mL volumes) in a vaccine adjuvanted with Freund's complete adjuvant. The birds are vaccinated with three injections administered at approximately two week intervals. Under veterinary supervision, vaccinations are delivered to chickens intramuscularly in the deep pectoral muscle. Booster injections are administered on the contra-lateral side.

Antibody titers. Blood is drawn from each bird at the time of each of the three injections, and about three weeks following the last injection. Serum antibody titers (IgG) are determined using an enzyme linked immunosorbant assay (ELISA). Adjacent wells of a microwell plate are coated with 2 µg aZP, and incubated for 6 hours. The wells are then blocked with 5% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in TBST (Tris-buffered saline + 5% Tween-20) and incubated overnight. Wells are then loaded with the primary antibody (i.e., avian serum) in TBST at a 1:500 and 1:1,000 dilution and incubated for 4 hours. The wells are then washed and loaded with 50 µl of the secondary antibody (rabbit anti-chicken IgG) and incubated for 2 hours. Color change is observed after the addition of *p*-nitrophenyl phosphate for 30 minutes and the reaction is terminated by the addition of 3 M NaOH. The optical density is read at a 405-492 nm range on a Spectramax spectrophotometer. The chickens' pre-immune serum serves as the negative control.

Egg laying. Eggs are counted beginning on the day of the first injection to evaluate reduction in egg production, providing direct evidence of fertility impairment.

The complete disclosure of all patents, patent applications, and publications cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. The invention is not

limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.